INKJET GENE PRINTING: A NOVEL APPROACH TO ACHIEVE GENE MODIFIED CELLS FOR TISSUE ENGINEERING

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ABSTRACT

In this study a novel method of simultaneous gene transfection and cell delivery based on inkjet printing technology is described. Plasmids encoding green fluorescent protein (GFP) were co-printed with living cells (porcine aortic endothelial (PAE) cells) through the ink cartridge nozzles of modified commercial inkjet printers. Agaorse gel electrophoresis analysis showed there was no obvious structural alteration or damage to these plasmids after printing. Transfection efficiency of the printed cells, determined by GFP expression, was over 10% and post-transfection cell viability was over 90%. We showed that printing conditions, such as plasmid concentration, cartridge model, and plasmid size influenced gene transfection efficiency. Moreover, genetically modified PAE cells were accurately delivered to target sites within a three dimensional fibrin gel scaffold and expressed GFP *in vitro* and *in vivo* when implanted into mice. These results demonstrate that inkjet printing technology is able to simultaneously transfect genes into cells as well as precisely deliver these cell populations to target sites. This technology may facilitate the development of effective cell-based therapies by combining gene therapy with living cells that can be delivered to target sites.

1. INTRODUCTION

In the interdisciplinary fields of tissue engineering and regenerative medicine, powerful new therapies are being developed to address structural and functional disorders of the human body by utilizing a combination of living cells and biological scaffolds as engineering materials (Griffith; Naughton 2002). The main goal of these therapies is to restore normal tissue function. In instances where normal tissue cannot be engineered with available cell types or enhanced cellular function is required to achieve a desired therapeutic outcome, alternative approaches such as growth factor supplementation, macromolecule treatment, or gene modification may be required. Moreover, in tissue engineering the transfection or delivery of functional genes into target cells to facilitate the formation of functional tissues and organs is becoming vital (Shea et al. 1999).

In various areas of research, gene modification techniques have been used to improve cell and tissue function. Although there are established methods for delivering genes into cells, such as viral transfection (Hendrie; Russell 2005), microinjection (Derouazi et al. 2006), electroporation (Isaka; Imai 2007), and liposome mediated transfection (Imitola et al. 2004), the application of these techniques to tissue engineering has been difficult due to either significant viral toxicity (e.g. transfection using viral vectors), low cell viability (e.g. electroporation), or low transfection efficiency (e.g. microinjection and liposome mediated method). In addition, the existing techniques require pre-processing of the cells prior to building new tissues for therapy. Therefore, there is a need to develop methods to effectively and efficiently transfect cells with specific genes during the tissue building process without compromising cell viability. This can be achieved by combining transfection and cell delivery using one platform for tissue engineering applications.

Inkjet printing technology employs the rapid creation and release of fluid droplets in combination with precise deposition on a substrate. Recently, the application of inkjet printing to tissue engineering has attracted much attention for its novelty and its ability to produce patterned biomaterials and complex structures containing living cells (Nakamura et al. 2005; Roth et al. 2004; Xu et al. 2005; Xu et al. 2004; Xu et al. 2006). In particular an innovative tissue fabrication method based on this technology known as “organ printing” has been proposed (Boland et al. 2003). Organ printing using the inkjet technology enables the delivery of multiple cell types and scaffolds to specific target regions for the efficient construction of functional tissues (Boland et al. 2006; Boland et al. 2007).

Droplet formation and ejection in thermal inkjet printing entails elements of high heat (up to 300° C) and velocity-induced sheer stress (up to 10 ms^{-1}) (Okamoto et al. 2000). Due to the short time required for nozzle firing (< 20 μs), cells survive passage through the printer nozzles and remain physiologically and functionally normal (Xu et al. 2006). During printing, transient distortion of the cell membrane with associated alteration of permeability may occur. It is well-established that brief application of electric fields or hydrodynamic pressure leads to changes in cell membrane permeability that allow the introduction of DNA and other macromolecules into cells (Wells 2004). With this in mind, we hypothesized that inkjet printing of cells can cause temporary disruption of the cell membranes and

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facilitate intracellular delivery of plasmid vectors. In this study we report simultaneous transfection with a plasmid vector and delivery of cells into two and three dimensional tissue constructs using an inkjet printer. Plasmids containing the reporter gene GFP were co-printed with porcine aortic endothelial (PAE) cells. We established that genetically modified PAE cells expressed the transfected GFP gene *in vitro* and *in vivo*. This demonstrates that this new technology can be used to develop engineered tissue for regenerative medicine applications.

2. MATERIALS AND METHODS

2.1 Cell cultures and staining

An established porcine aortal endothelial (PAE) cell line was used in this study(Schweigerer et al. 1987). Passages 15, 16 and 17 of the cell line were used for gene transfection. PAE cells were maintained in F12 medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO), 100 IU of penicillin and 100 mg/ml of streptomycin and incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.2 Preparation of plasmid DNA

The plasmids pmaxGFP (Amaxa GmbH, Germany) and pIRES-VEGF-GFP (BD Biosciences, Bedford, MA) encoding the cDNAs of jellyfish *Aequorea victoria* green fluorescent protein, driven by the cytomegalovirus (CMV) early immediate promoter, were amplified in DH5α strain of *Escherichia coli*. Plasmid was released by alkaline lysis and purified by ion exchange column chromatography using QIAfilter Plasmid Maxi Kit (Qiagen Inc., Valencia, CA).

2.3 Preparation of inkjet printers and "bio-paper" substrates

HP DeskJet 692C and 550C printers as well as HP 51626a and 51629a ink cartridges were modified as previously described(Xu et al. 2006). Printer cartridges ware rinsed with ethanol and sterile water and placed into the printer assembly. The whole assembly was kept in a laminar flow hood under UV light overnight prior to use. The printer was programmed to print a square using Microsoft PowerPoint®. Bio-paper substrates were prepared from rat-tail Type I collagen gels using a previously reported protocol(Xu et al. 2005). Briefly, rat-tail Type I collagen (BD Biosciences, Billerica, MA) was diluted to a concentration of 1.0 mg/ml with chilled PBS (Phosphate Buffered Saline). After adjusting to about pH 7.4, 1.5 ml aliquots of the mixture were dispersed onto coverslips and cured in an incubator for 3–5 h. Once the gel set, the collagen bio-paper was ready for use.

2.4 Inkjet printing of plasmid DNA and gel electrophoresis

To determine the effect of the inkjet printing on plasmid DNA integrity, pmaxGFP and pIRES-VEGF-GFP were diluted in Nucleofector solution (Amaxa) to a concentration of 1ug/ul. The plasmid suspensions were loaded into the cartridge and printed into a 1.5ml centrifuge tube with a HP 692C printer and HP 51629a ink cartridge. 0.5ul samples of pre- and post-printed plasmid were electrophoresed in a 1% agarose gel containing ethidium bromide and visualized by UV transillumination.

2.5 In vitro cell printing and gene transfection

Plated cultures of PAE cells were trypsinized and pelleted by centrifugation. The cells were subsequently re-suspended in the suspension buffer supplied with the nucleofection kit (Amaxa) at a density of 1.5 - 2 × 10^6 cells/ml. The plasmids were added to the cell suspension at concentrations ranging from 0.1μg/μl to 2μg/μl. Print suspensions containing PAE cells and plasmids were loaded directly into ink cartridges. The mixture was printed onto collagen gel substrates. After a 30 min incubation at 37°C in a humidified 5% CO_2 atmosphere the printed collagen gels were carefully placed in culture dishes of media to avoid disrupting the printed cell patterns. As a control, PAE cells and plasmid were mixed together and manually seeded onto collagen gel substrates with the same cell density and plasmid concentrations used in the printed group. Expression of the transfected genes and cell growth were monitored daily via light and fluorescent microscopy.

Some measures were applied to prevent cell agglomeration and sedimentation in the cell suspension loaded into the print cartridge. Before loading, the cell print suspension was filtered with 40-um strainer and shaken gently and thoroughly to avoid clumps and ensure uniform distribution of the cells. The actual printing was performed immediately upon loading so that sedimentation time was minimized. Furthermore, the movement of the cartridge throughout the printing procedure provided a continuous and gentle shake to cell suspension loaded in the cartridge. In this study, we printed a small amount of cell suspension, about 500 ul each loading. It only took a few minutes to print out such a small amount of cell suspension. The short printing timeframe combined with cartridge's shake can reduce the possibility of cell agglomeration and sedimentation.

2.6 In vitro gene transfection using liposome and electroporation

For Lipofectamine 2000 (Invitrogen) mediated transfection, PAE cells were seeded on a 24-well plate at a density of 2×10^5 cells per well the day before transfection. The manufacturer's transfection protocol was followed. Briefly, 0.8ug of pmaxGFP (Amaxa) and the equivalent amount of Lipofectamine 2000 reagent were each added to 50µl of serum-free F12 medium. After equilibration for 5 min at room temperature (RT) the solutions were mixed and incubated for 20 min at RT. Subsequently the DNA/liposome complex was added to the cells in the 24-well plate and maintained for up to 48 h.

The Basic Nucleofector Kit for Primary Mammalian Endothelial Cells (Amaxa) was used for nucleofection. Two standard electroporation programs (W-023 and Y-022) recommended by the kit manufacturer were tested. Both were designed for transfection of primary endothelial cells from porcine aorta. Program W-023 showed better results in terms of viability and transfection efficiency for the PAE cells and was used for further experiments. The transfection was performed according to the manufacturer's protocol for endothelial cells. After trypsin treatment, detached cells were adjusted to a concentration of 1.5×10^6 cells /ml with culture medium. Cells suspensions were centrifuged and media was removed. Subsequently cells were re-suspended in 100 µl of Nucleofection buffer together with plasmid DNA. After the electrical pulse, 500 µl of F12 medium (Gibco) containing 10% FBS were immediately added to dilute the Nucleofection solution. Treated cells were seeded on 60mm dishes containing pre-warmed culture medium and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 hours before further analyses.

2.7 Cell viability

Cell viability following each transfection method was evaluated by using the tetrazolium compound (MTS) assay (Sigma-Aldrich) according to the manufacturer's protocol. 200ul of reagent was added per ml of media in the transfected and control samples. After the samples were incubated for 2 hours in the dark at RT, the absorbance at 540 nm was measured using a spectrophotometer. The percentages of viable cells resulting from the three different transfection methods were estimated by calculating the ratio between the absorbance of the transfected samples to those of the non-transfected samples.

2.8 Transfection efficiency measurements

After 24-48 hours of culture, the transfected samples were thoroughly washed with PBS to remove the lysed and non-adherent cells, and fixed with 4% paraformaldehyde solution. DAPI stain (10 mg/ml) was used to evaluate the total number of viable cells in the cultures. For cells transfected with GFP expression vectors (pmaxGFP and pIRS-VEGF-GFP) the GFP-positive cells were counted. Cells were counted under 100× magnification using an inverted Zeiss fluorescent microscope. Four fields were randomly selected in every well, and at least 4 wells were counted for each sample. The transfection rate of the viable adherent cells was calculated from the ratio of GFP-positive cells and DAPI-positive cells. The total transfection efficiency was estimated by multiplying the transfection rate by the percentage post treatment viability.

2.9 In vivo direct printing and gene transfection

In vivo gene transfection induced by inkjet printing was assessed by direct printing of the PAE cells and plasmid DNA into the subcutaneous tissues of athymic mice. Fibrinogen (5 mg/ml dissolved in PBS) (Sigma) and thrombin (5 IU/ml in 40 mM $CaCl_2$) (Sigma) were alternately printed into the subcutaneous tissues to generate a fibrin gel scaffold, then the PAE cells and pmaxGFP plasmid were co-printed onto the fibrin scaffold. This procedure was repeated twice, resulting in a three dimensional cuboid of fibrin (1cm×0.5cm×0.2cm) which contained the transfected cells. After 1 week of implantation, the *in vivo* printed fibrin cube was retrieved and immediately examined under a fluorescent microscope. As a control, fibrin gel cuboids were fabricated by manually seeding PAE cells into fibrin gels before implantation into mice. All animal experiments were performed according to ACUC protocols at Wake Forest University Health Sciences.

3. RESULTS

3.2 Gene transfection in vitro

Inkjet mediated gene transfection was achieved using commercial Hewlett Packard (HP) desktop printers (models 692C and 550C) and ink cartridges (HP 51629a or 51626a) with previously described modifications(Xu et al. 2006). To determine whether plasmid integrity was maintained after passage through the printer nozzles, solutions containing plasmid (pmaxGFP and pIRES-VEGF-GFP, at a concentration of 1µg/µl) were printed into 1.5ml tubes and examined by electrophoresis. Agarose gel electrophorosis analysis showed that the supercoiled or nicked plasmid content of the printed samples was similar to that of the controls (data not shown). In addition, background DNA smearing was not evident, which indicated that the plasmids were not structurally altered by the printing process.

To test the efficacy of inkjet-mediated transfection, porcine aortal endothelial (PAE) cells (Schweigerer et al. 1987) were mixed with the plasmid expression vector pmaxGFP, which encodes bacterial green fluorescent protein (GFP) under cytomegalovirus (CMV) promoter control. The mixture was either seeded manually (not printed) or delivered by direct printing onto a collagen substrate and the samples were evaluated for viability and GFP expression. The PAE cells co-printed with plasmid DNA retained their characteristic morphology (Fig. 1A) and a subpopulation of the cells showed strong green cytoplasmic fluorescence, demonstrating successful transfection with pmaxGFP plasmid (Fig. 1B). Moreover, strong GFP expression in the printed PAE cells was consistently observed over a 10-day period. In contrast, the non-printed control cells failed to express the transfected gene (Fig. 1C, D).

cells (Fig. 1E). Over 90% of the cells survived the printing and transfection process. This concurs with our and other group's previous published reports (Nakamura et al. 2005; Xu et al. 2005), and reconfirms our previous conclusion that the inkjetting process results in minimal damage to the cells(Xu et al. 2006). Up to 12.8% of the printed cells were successfully transfected and expressed the plasmid encoded reporter genes. These results indicate that the physical process of inkjetting, which involves heat and shear shock, can facilitate the entry of plasmid into the printed cells. However, the exact mechanism of inkjet mediated transfection needs further investigation. As shown in Figure 1F, the total efficiency of the inkjet transfection with pmaxGFP (12.8%) was significantly lower than that of the electroporation-based method, which had an efficiency of 32.3%. However, it was higher than that of the liposome-based method, which had an efficiency of 10.6%.

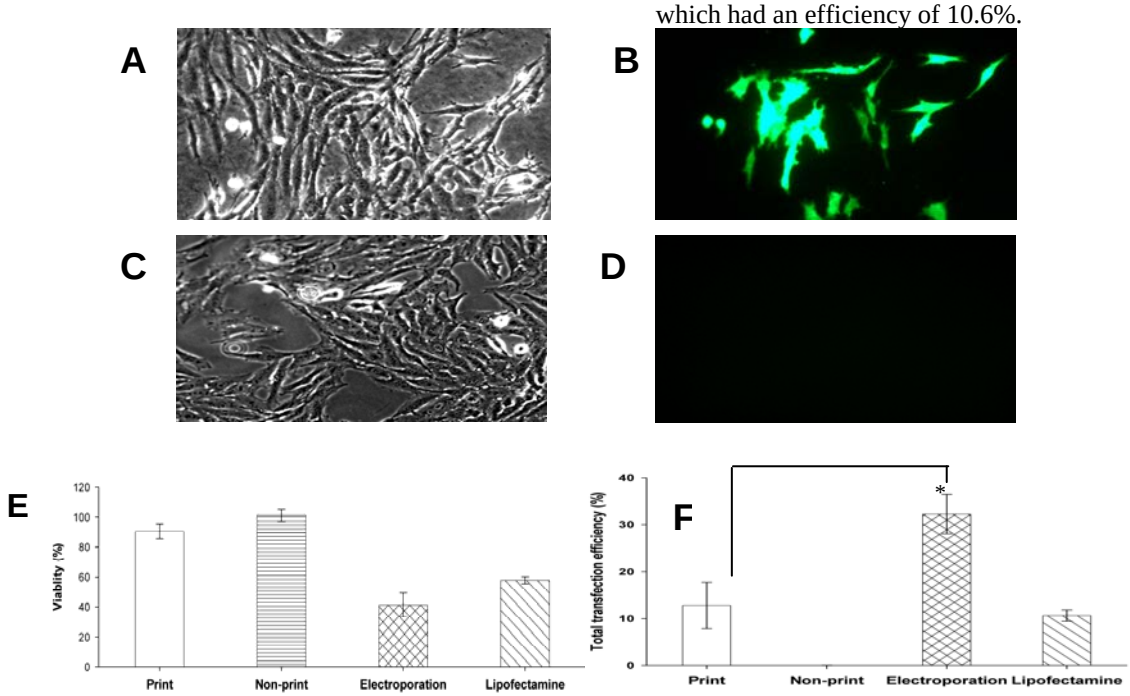

Fig. 1. *In vitro* gene printing. (A-D): Morphologies of printed and non-printed cells. The printed PAE cells exhibited normal morphology on the collagen gels 2 days after printing (size bar 5μm) (A). A number of cells in the printed samples exhibited strong cytoplasmic green fluorescence (B). The non-printed cells (controls) also showed normal morphology (size bar 5μm) (C), but were not detectably transfected with the GFP plasmid(D). (E-F): Viability and transfection efficiency comparison of the inkjet transfection method with other common gene transfection methods. Compared with the common chemical (Lipofectamine) or electroporation (Nucleofection) associated method, the inkjet transfection method had higher cell viability after transfection (E). The total transfection efficiency (combined with viability) of the inkjet method is lower than that of the Nucleofection method but higher than the Lipofectamine method (F).

Next, the inkjet printing method was compared to other chemical and physical transfection methods commonly used in tissue engineering applications. Lipofectamine 2000, a common liposome based reagent, and Nucleofection, a proprietary electroporation approach, were employed to transfect plasmids into PAE cells. When compared with the two common chemical and physical methods, inkjet printing showed significantly higher cell viability of the processed living

3.3 The influence of different printing conditions on gene transfection

The effect of different printing parameters on transfection efficiency was evaluated. First the effect of plasmid concentration in the print suspension was tested. As shown in Figure 2a, transfection efficiency was dependent on plasmid concentration with maximum

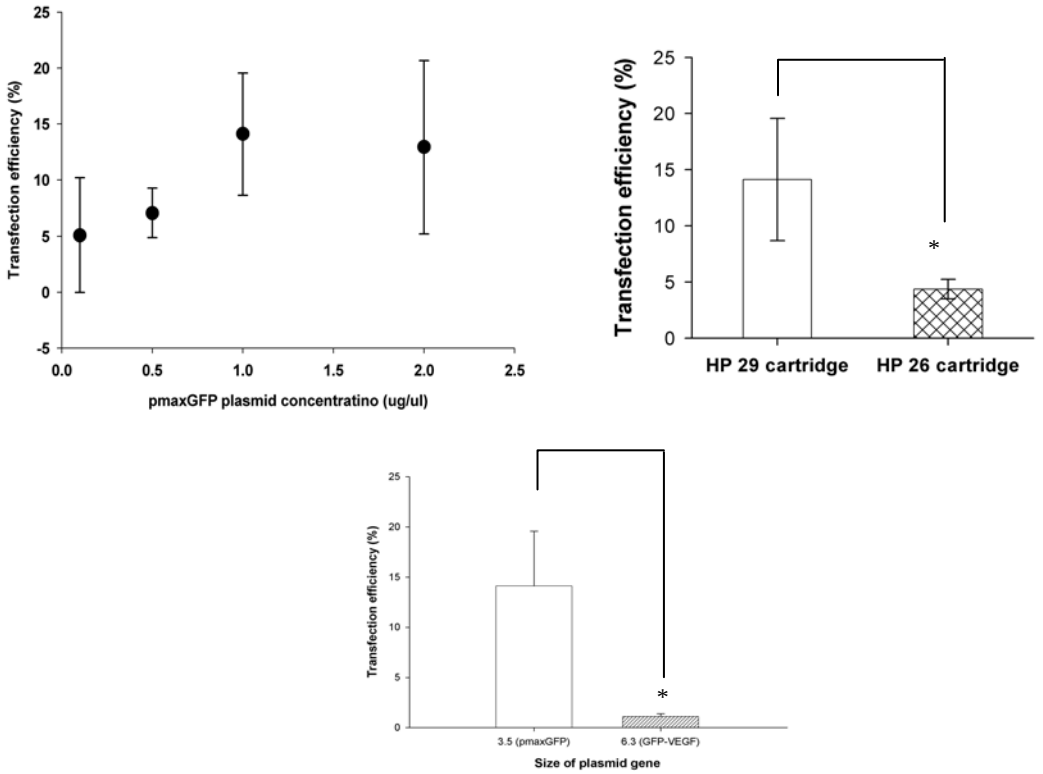

Fig 2. Effects of the printing parameters and conditions on gene transfection. The higher plasmid concentrations exhibited higher transfection efficiency (a). The use of the HP 29 ink cartridge caused higher gene expression than the use of the HP 26 ink cartridge (b). Compared with the larger plasmid, pIRES-VEGF-GFP, the smaller pmaxGFP plasmid exhibited higher transfection efficiency of the PAE cells (c).

efficiency at 1.0μg/μl. Two different ink cartridges, HP 51629a and 51626a, were tested in this study. It was found that higher gene transfection efficiency was obtained using the HP 51629a cartridge, 14.1% versus 4.3%. (Figure 2b). This may be due to higher sheer stress developed during printing as the nozzle size of HP 51629a cartridge is smaller than that of the HP 51626a cartridge(Cartagena Personal communication). In addition, the role of plasmid size on transfection efficiency was also tested. Transfection with the small pmaxGFP plasmid (3.2kb) was dramatically more efficient (14.1%) when compared to the larger plasmid, pIRES-VEGF-GFP (6.3 kb), which transfected with 1.1% efficiency (Figure 2c).

3.4 In vivo gene transfection

The feasibility of applying inkjet mediated plasmid transfection to *in vivo* applications was also investigated. A layer of fibrin gel was formed by directly printing fibrin glue solutions into the subcutaneous tissues of athymic mice. PAE cells mixed with pmaxGFP plasmid (1μg/μl) were subsequently printed directly onto the pre-formed fibrin gel. By repeating this process twice, cuboids (1cm×0.5cm×0.2cm), made up of alternating layers of fibrin gel and cells, were formed *in situ* directly under the subcutaneous tissues in the test mice. One week after implantation the fibrin gel implant was retrieved and its shape was found to be conserved (Figure 3a-b). The printed cells expressing the GFP gene were clearly seen within the fibrin gel under UV fluorescent microscopy (Figure 3c). However, little, if any, GFP gene expression was observed in the non-printed control group in which the implant consisted of a fibrin laminate containing manually seeded cell samples.

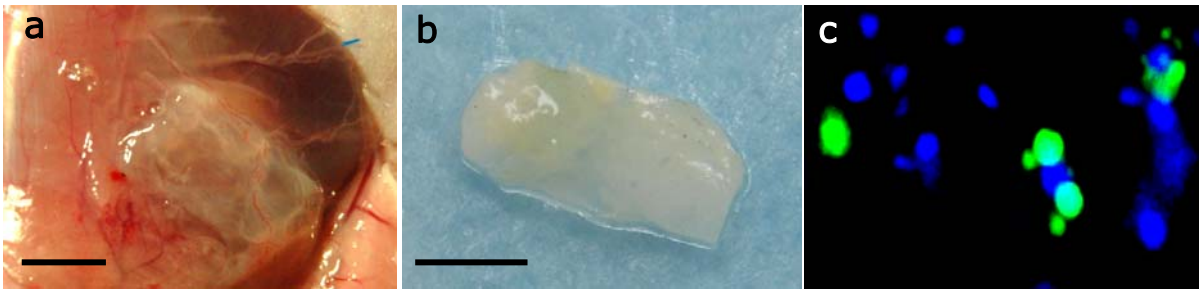

Fig 3. *In vivo* inkjet printing and gene transfection. (a) Retrieval of the fibrin gel implant from the subcutaneous tissues of the nude mouse 1-week after implantation. The *in vivo printed* fibrin cube was clearly visible within in the subcutaneous tissues of the mouse and vasculature was present in the fibrin cube (Size bar 5mm). (b) Gross examination of the retrieved fibrin cube showed the rectangle form (1cm×0.5cm×0.2cm) which matched with the pre-designed pattern used for direct *in situ* printing (Size bar 5mm). (c) Fluorescent microscopy of the cells within the fibrin cube. GFP expression was found among a number of cells entrapped within the fibrin cube (green). The nuclei of all cells trapped within the cube were visualized by DAPI staining (blue).

4. DISCUSSION

In this study we demonstrate a direct, noninvasive, nonviral, and efficient gene transfection system based on inkjet printing. As hypothesized, naked plasmid DNA encoding a reporter gene, GFP, was successfully delivered into endothelial cells (PAE cells) during inkjet printing and the transfected GFP genes were expressed both *in vitro* and *in vivo*. Transfection is thought to be mediated temporary disruption of cell membranes that facilitates the intracellular delivery of plasmid vectors. A postulated mechanism for inkjet mediated gene transfection is summarized schematically in Figure 4. When cells and plasmid pass through the ink channels of the printhead during the printing process, the high shear stress and heat generated upon nozzle firing may cause temporary micro-disruption of the cell membrane. Plasmids could then pass into the cells via transient pores created by this disruption. Further work is needed to define the exact mechanism of inkjet-mediated transfection. This is the first report that physical or mechanical forces present during the process of inkjet-mediated cell printing can allow gene transfection. We have named this phenomenon "jetoporation".

It is important to note that inkjet mediated gene transfection is less efficient than electroporation. A possible reason for this is that the energy levels present during jetoporation are relatively low compared to those present during electroporation. Electroporation is a widely studied gene delivery method that employs an exponentially decaying external field pulse of high intensity (KV/cm) and short duration (up to about 100 μsec) to cause a reversible voltage induced breakdown of the cell membrane (Zimmermann et al. 1974). Due to the high energy levels present during electroporation, this method results in high transfection efficiencies in well-established cell lines (Hamm et al. 2002). In this study when electroporation was used, a transfection efficiency of over 70% was obtained in PAE cells. However, the high energy transfection conditions used for electroporation usually compromise cell viability. For example, in this study, over 60% cells were lysed during the electroporation process (Figure 1f). Taking this into account, the total transfection efficiency for electroporation was decreased to approximately 32%. In contrast, the inkjet printing method, which uses low energy conditions, did not have obvious adverse effects on the structure of the printed plasmids (Figure 1a) and over 90% of the PAE cells survived the printing (transfection) process (Figure 1f). The low energy conditions may result in lower levels of cell membrane permeability which lowers transfection efficiency but increases cell viability. However, the low energy inkjetting conditions may limit efficient entry of large plasmids into the cells (Figure 2c) because only small micro-pores could be opened in the cell membrane. Due to the high cell viability obtained with the inkjet mediated method, it resulted in a comparatively high transfection rate of over 10% for PAE cells in this study. In addition to causing immediate necrosis of most cells subjected to high electrical field strengths, electroporation may also induce damage to the DNA in the surviving cells. This can lead to abnormal function and delayed apoptosis of these cells (Hofmann et al. 1999). This has not an issue with cell printing. Further research and development of the inkjet based gene delivery system is needed in order to significantly increase transfection efficiency. In particular, specific printing parameters and conditions such as temperature, firing frequency and structural design of the cartridge ink channel need to be explored, as these parameters and conditions were shown to specifically affect transfection efficiency in this study (Figure 3).

An obvious advantage of the inkjet mediated transfection is the possibility of the simultaneous transfection and delivery of cells into two and three dimensional tissue constructs during tissue fabrication. Currently, transfection of donor cells with specific genes has been successfully applied in tissue engineering applications to provide sustained and efficient delivery of inductive molecules (e.g. cytokines, growth factors, etc) in order to enhance tissue formation in engineered constructs(Shea et al. 1999). To better mimic the natural

signaling processes that occur in developing tissues, it may be crucial that these factors are expressed in a specific spatial and temporal pattern (Dang; Leong 2006). In principle, this could be achieved if transfected cells are placed at predetermined locations within engineered tissues. However, conventional gene transfection methods, such as electroporation, viral transfection, and microinjection require that cells be transfected prior to their use for tissue construction. In addition, traditional tissue building methods lack an effective mechanism to deliver the transfected cells to specific target sites. By combining the ability of the inkjet printer to target multiple cell types to specific sites (Boland et al. 2006) with the simultaneous gene delivery process described here, efficient gene transfection and precise cell delivery can be integrated into one platform. As demonstrated in this study the printed transfected PAE cells can be assembled into 3D fibrin gel constructs (cuboids) *in vivo* and the GFP transgenes are expressed (Figure 3). In comparison with other tissue engineering and gene transfection approaches this new system of simultaneous gene transfection and cell delivery for tissue fabrication is less complicated, less expensive, and less time consuming. In addition, the possibility of contamination is reduced.

5. CONCLUSION

This study demonstrates that inkjet technology can be used to deliver genes into living cells for tissue engineering applications. This technique can simultaneously transfect genes into cells and deliver these cells to precise target sites. Genes transfected into cells using this approach were expressed both *in vitro* and *in vivo*. Furthermore, this combination of direct *in vivo* printing and gene transfection opens the possibility of fabricating three dimensional structures within the body that express essential regulatory factors required to drive development, differentiation and functional tissue regeneration.

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